



SHORT REPORT

CDX2 is mutated in a colorectal cancer with normal APC/ β -catenin signaling

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The majority of human colorectal cancers have elevated β -catenin/TCF regulated transcription due to either inactivating mutations of the APC tumor suppressor gene or activating mutations of β -catenin. Surprisingly, one commonly used colorectal cancer cell line was found to have intact APC and β -catenin and no demonstrable β -catenin/TCF regulated transcription. However, this line did possess a truncating mutation in one allele of CDX2, a gene whose inactivation has recently been shown to cause colon tumorigenesis in mice. Expression of CDX2 was found to be induced by restoring expression of wild type APC in a colorectal cancer cell line. These findings raise the intriguing possibility that CDX2 contributes to APC's tumor suppressive effects.

Keywords: CDX2; APC; colorectal cancer; mutation; regulation

Mutations in the adenomatous polyposis coli (*APC*) gene initiate the vast majority of human colorectal tumors (Kinzler and Vogelstein, 1996) but the functional consequences of such mutations at the biochemical level were until recently poorly understood. An important advance was made with the discovery that the *APC* gene product inhibits β -catenin/TCF regulated transcription (CRT) (Korinek *et al.*, 1997; Morin *et al.*, 1997). This inhibition is likely mediated by the binding of β -catenin to APC (Rubinfeld *et al.*, 1993; Su *et al.*, 1993), which facilitates phosphorylation of β -catenin by GSK-3 β (Rubinfeld *et al.*, 1996), and leads to its degradation through ubiquitination-dependent proteolysis (Munemitsu *et al.*, 1995; Aberle *et al.*, 1997; Orford *et al.*, 1997). APC thus effectively regulates the cellular levels of β -catenin and consequently the formation of active transcription complexes between β -catenin and Tcf-4. Consistent with this model, alterations of β -catenin that render it refractory to regulation by APC were identified in a number of tumors with wild-type APC (Morin *et al.*, 1997; Rubinfeld *et al.*, 1997; Sparks *et al.*, 1998), and it was shown that these β -catenin mutations as well as inactivating mutations of *APC*

result in constitutive CRT (Morin *et al.*, 1997). Accordingly, previous work had shown that all colorectal cancer cell lines tested had constitutive CRT (Korinek *et al.*, 1997), suggesting that CRT deregulation is a key event in colorectal cancer initiation.

The present study originated from the surprising observation that CRT is absent in RKO, a widely studied colorectal cancer cell line (Brattain *et al.*, 1984). We subsequently found that RKO contains a mutant *CDX2* gene, a gene recently implicated in intestinal tumorigenesis in mice (Chawengsaksophak *et al.*, 1997), and that *CDX2* transcription is induced by APC. These results suggest a connection between APC and *CDX2* in colorectal cancers and raise the possibility that *CDX2* is one of the mediators of APC's tumor-suppressing activity.

β -catenin regulated transcription activity in cancer cell lines

Constitutive activation of β -catenin regulated transcription has been reported in all colorectal cancer cell lines tested, a feature that distinguishes them from cancer cell lines derived from other tissue types (examples in Figure 1). CRT is typically measured using luciferase reporters called TOPFLASH and FOPFLASH, which contain promoters with several copies of a normal or mutant version of a TCF-response element (Korinek *et al.*, 1997), respectively. Activation of CRT results in a high ratio of TOPFLASH to FOPFLASH luciferase activity. Surprisingly, when we measured CRT in RKO, we found that the levels of luciferase activity were similar with both TOPFLASH and FOPFLASH reporters (data not shown). However, we also observed that the levels of FOPFLASH reporter activity were significantly higher in RKO than in the other cancer cell lines tested. We were therefore unable to conclude that RKO lacked constitutive CRT activity, as it was possible that it was simply masked by high background activity of the FOPFLASH control in this particular cell line. To distinguish between these two possibilities, we constructed a new pair of CRT reporter vectors (TCF-Luc and TCF*-Luc) that displayed much lower background activities in all lines tested (JY and LTC, unpublished observations). We then repeated our analysis of CRT activity in RKO using these vectors and demonstrated that RKO

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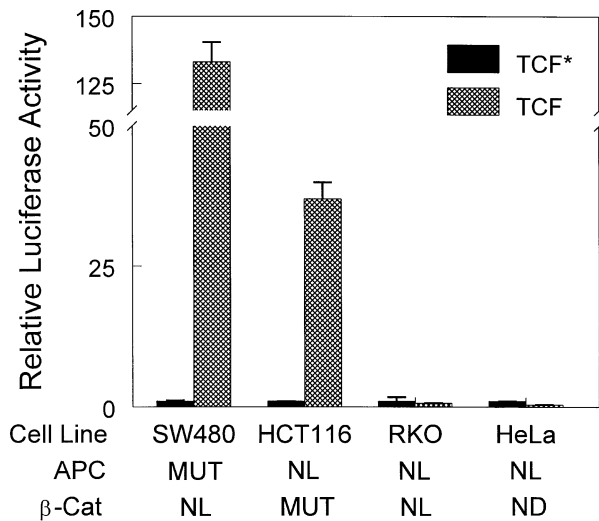


Figure 1 CRT activity in cancer cell lines. The indicated cell lines were transfected with luciferase reporter constructs containing three consensus (TCF-Luc) or mutant (TCF*-Luc) TCF-binding sites, plus a CMV-driven β -galactosidase (β -gal) reporter to control for transfection efficiency. Twenty-four hours later, the cells were collected and luciferase and β -gal activities determined. Bars represent average luciferase activities normalized to the values obtained with TCF*-Luc. SW480, HCT116 and RKO are human colorectal cancer (CRC) lines. CRT activity in HeLa, a human cervical cancer line, is typical for non-CRC lines (Korinek *et al.*, 1997). The status of APC and β -catenin in each line is indicated (NL, no abnormalities detected; MUT, mutant; ND, not determined). Details of the construction of the TCF-Luc and TCF*-Luc reporters are available upon request. The pCMV β vector (Clontech) was used as the CMV-driven β -galactosidase (β -gal) reporter control. Cell lines were maintained in McCoy's medium (Life Technologies, Gaithersburg, MD, USA) supplemented with 10% fetal bovine serum and antibiotics. Transfections were performed on 24-well tissue culture plates when cells were 40–60% confluent using Lipofectamine (Life Technologies), or FuGENE6 (Boehringer-Mannheim) according to the manufacturer's instructions. Cells were collected and luciferase and β -galactosidase activities determined using the Luciferase Assay Reagents kit (Promega) and the Aurora GAL-XE Kit (ICN), respectively

lacked constitutive β -catenin regulated transcription (Figure 1). We further determined that this was not due to an intrinsic inability to activate TCF-responsive elements, since TCF-Luc, but not TCF*-Luc, could be activated in RKO by co-transfection with a plasmid encoding β -catenin/S33Y (Morin *et al.* 1997), a constitutively active mutant β -catenin (data not shown).

Genetic analysis of RKO

Because exceptional cases are often of considerable heuristic value, we decided to investigate RKO in greater detail. As expected from the above results, mutational analyses of the β -catenin (*CTNNB1*), *TCF-4* and *APC* genes revealed no CRT-activating alterations (Sparks *et al.*, 1998 and Figure 2). We thus suspected that mutation of a downstream component of the pathway might have substituted for APC inactivation in this line. In particular, we were intrigued by the report that *Cdx2*^{+/-} mice develop multiple adenomatous polyps in the colon (Chawengsaksophak *et al.*, 1997). As multiple intestinal tumors in mice had previously been routinely observed only in

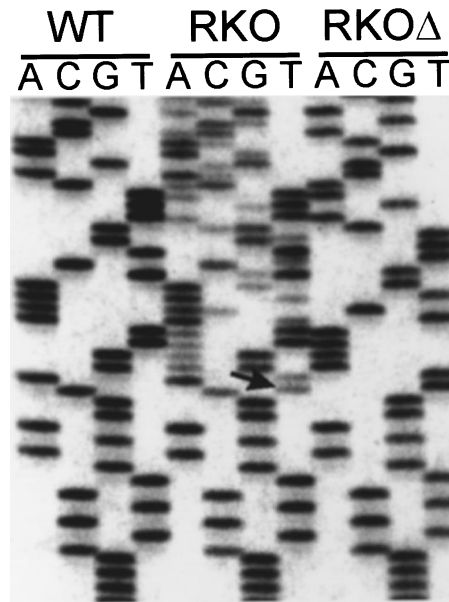


Figure 2 Mutational Analysis of *CDX2* and the APC pathway in RKO. Autoradiograph of a sequencing gel showing the 4 bp deletion in the cDNA of RKO. Sequencing reactions performed directly on the RT-PCR product (RKO) and on two clones are shown. Cloning of the RT-PCR product yielded clones with wild-type (WT) or mutant (RKO Δ) sequence. The complete coding region of *CDX2* was amplified using primers CDX2-0039F and CDX2-1047R. The PCR products were purified by isopropanol precipitation and sequenced with primers CDX2-0048F, CDX2-0252F, CDX2-0413F, CDX2-0604F and CDX2-1035R (Table 1) as described below. APC, β -catenin, and *TCF-4* did not display mutations (data not shown). Western blot analysis of APC in RKO was performed as previously described (Smith *et al.*, 1993) and revealed apparently full-length APC protein. Since it was possible that mutations resulting in very late truncations could be missed by Western blotting, codons 2230 through the carboxyl-terminus of APC were analysed by direct sequencing as described (Powell *et al.*, 1992). For β -catenin analysis, a genomic PCR fragment including codon 1 in exon 2 to codon 90 in exon 4 and encompassing the N-terminal regulatory region was amplified as described (Sparks *et al.*, 1998). PCR products were gel-purified and sequenced directly using ThermoSequenase (Amersham) and ³³P-labeled ddNTP's (Amersham) according to the manufacturer's instructions. The *TCF-4* transcript was amplified by RT-PCR in two segments and its entire coding region excluding codons 1–16 was sequenced. Purification of genomic DNA and RNA, PCR, and RT-PCR were performed as described (Riggins *et al.*, 1995)

Apc^{+/-} animals (Su *et al.*, 1992, Fodde *et al.*, 1994), this observation raised the possibility that *CDX2* might play a role in the initiation of human colorectal tumors as well. Sequencing of the coding region of *CDX2* indeed revealed a heterozygous 4 base-pair deletion (AGGCAGG to AGG) within the sequence corresponding to helix 3 of the *CDX2* homeodomain (Figure 2). The predicted abnormal protein lacked the carboxyl-terminal 85 amino acids, including a major part of the DNA-binding domain. These were substituted with 45 new residues and the protein was predicted to be transcriptionally inactive. Interestingly, no mutation in the remaining allele of *CDX2* was detected in RKO cells. Similarly, no loss or alterations of the wild-type *Cdx2* allele were found in colon tumors developing in *Cdx2*^{+/-} mice (Chawengsaksophak *et al.*, 1997).

To test the hypothesis that the mutation in *CDX2* functionally altered its transcriptional activation

capabilities, we constructed plasmids encoding wild-type (CMV-CDX-WT) or mutant (CMV-CDX-MUT) *CDX2* genes under control of a CMV promoter-enhancer, as well as reporter vectors containing Luciferase under control of a CDX2-responsive promoter (SI-Luc) (Suh *et al.*, 1994). Co-transfection experiments showed that CMV-CDX-WT, but not CMV-CDX-MUT could activate transcription of the reporter constructs (Figure 3), formally demonstrating that the *CDX2* mutation in RKO inactivated the gene product.

CDX2 regulation by APC

The occurrence of a *CDX2* mutation in a tumor cell line with wild-type APC and without CRT activity suggested that there might be a connection between APC and CDX2 in human cancer, and in particular that CDX2 might be one of the effectors of APC's tumor-suppressing activity. Alternatively, CDX2 and APC could both behave as tumor suppressor genes independently of one another (He *et al.*, 1997). To begin to distinguish between these two possibilities, we investigated whether APC could regulate the levels of *CDX2* RNA. HT29-APC and HT29-GAL are paired colorectal cancer cell lines that contain zinc-inducible wild-type APC or β -galactosidase genes, respectively (Morin *et al.*, 1996). The endogenous APC genes in HT29 cells are mutant. Upon Zinc induction, HT29-APC, but not HT29-GAL, express wild type APC, which inhibits the interaction between β -catenin and Tcf-4 and consequently downregulates CRT activity. Total RNA was collected from HT29-APC and HT29-GAL cells at different time points after Zinc induction (Morin *et al.*, 1996; He *et al.*, 1998) and RNase protection assays were performed on these samples with a probe derived from the 3'-untranslated region of *CDX2*. As shown in Figure 4, induction of APC was associated with a rapid and significant increase in the level of *CDX2* RNA.

Autoregulation of CDX2 expression

In addition to developing colonic tumors, Chawengsaksophak *et al.* (1997) reported that *Cdx2*^{+/-} mice suffer from a variety of developmental abnormalities that display a homeotic character. These fall into well-defined categories, but differ significantly from animal to animal, suggesting that they depend on a stochastic event. Furthermore, the remaining wild-type allele is not expressed in the mouse intestinal tumors that develop. How could loss of one allele of *Cdx2* result in reduced expression of the remaining, wild-type allele? We postulated that this would be possible if the level of expression of *Cdx2* was regulated, in part, by *Cdx2* itself, thereby operating through a positive feedback loop. To test this possibility, we constructed a reporter vector in which the luciferase gene was driven by a fragment of the human *CDX2* gene promoter (CDX-Luc). As shown in Figure 3, CDX-Luc could be specifically activated by wild-type CDX2, but not by the mutant CDX2 protein found in RKO cells, suggesting that the CDX2 protein indeed contributes to its own expression through autoregulation.

In summary, we have shown that the RKO cell line contains wild type APC, *CTNNB1* and *TCF4*

genes. RKO is the first human colorectal cancer cell line shown to lack constitutive β -catenin regulated transcription, and it contains an inactivating mutation of the *CDX2* gene. In this regard, it is interesting that Wicking *et al.* (1998) recently reported a case of a replication error-prone (RER+) colorectal cancer with mutations in both alleles of *CDX2*. As RKO is also mismatch repair deficient (Eshleman *et al.*, 1995), mutations in this cell line must be regarded with caution. However, the *CDX2* mutation in RKO was shown to functionally inactivate the gene product through experiments with two completely different reporters. We also show that restoring expression of full-length APC in a human colorectal cancer cell line can induce *CDX2* expression. Finally, we have shown that CDX2 can positively regulate its own expression. Taken together, the data suggest the following model for colon cancer initiation. In most cases, the normal down-regulation of β -catenin/TCF by APC is abrogated by mutations in either APC or β -catenin, leading to constitutive transcription of β -catenin/TCF-responsive genes and indirectly to a variety of gene expression alterations, including up-regulation of *c-MYC* (He *et al.*, 1998) and abnormally low levels of *CDX2* expression. It is important to note that, unlike *c-MYC*, the effects of APC on *CDX2* cannot be mediated directly through β -catenin/TCF since APC induces *CDX2* rather than repressing it. The link between APC and *CDX2*, even if mediated by the β -catenin/TCF

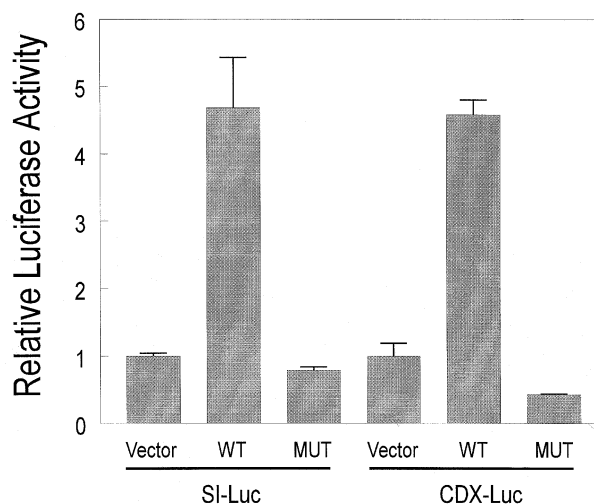


Figure 3 Transcription activity of wild type and mutant CDX2. SW480 cells were transfected with a luciferase reporter gene (0.5 μ g) under control of a fragment of the Sucrase-Isomaltase (SI-Luc) or the CDX2 (CDX-Luc) promoter, an effector construct (2.0 μ g) with CMV-driven wild-type (WT), mutant (MUT) or no (Vector) CDX2 genes, and CMV-driven β -galactosidase (β -gal) to control (0.5 μ g) for transfection efficiency. Forty-eight hours later, the cells were collected and luciferase and β -gal activities determined as described in Figure 1. The SI-Luc reporter was derived from TCF-Luc by replacing the *PstI-XbaI* fragment containing the TCF-binding sites with a CDX2-responsive fragment of the Sucrase-Isomaltase gene promoter. CDX-Luc was created from pGL3-Basic (Clontech) by inserting a 9 kb bp *XhoI* genomic DNA restriction fragment from the *CDX2* promoter, with its 3' end 200 bp downstream from the presumed transcription start site. Bars represent the average luciferase activities normalized to the vector control

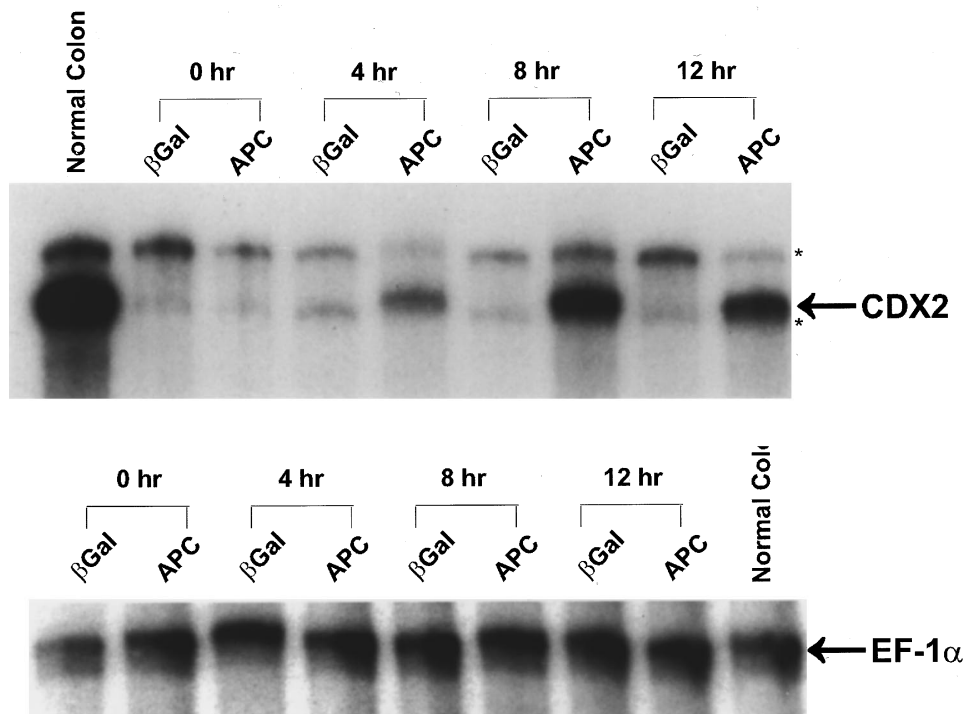


Figure 4 *CDX2* regulation by APC. RNA was purified from cells lines HT29-APC and HT29-GAL at the indicated times after induction. Twenty μg of total RNA from normal colon and 30 μg from each cell line were used in RNase protection assays (RPA) with probes derived from the 3'-untranslated region of the human *CDX2* gene (top) or *EF-1 α* (as a control). Asterisks indicate nonspecific bands. HT29-APC and HT29-GAL cells contain zinc-inducible *APC* and *β -gal* genes, respectively (Morin *et al.*, 1996). Cells were plated at 40–50% confluency, induced two days later with 120 μM ZnCl_2 and harvested at the indicated times after induction. Total RNA was prepared using Promega's RNA isolation kit following the manufacturer's instructions. A *Pst*I restriction fragment containing nucleotides 1018–1346 of *CDX2* cDNA (GENBANK accession number U51096) inserted into pZero (Invitrogen) in the anti-sense orientation respective to the T7 promoter was used as template to prepare the RNase protection probe. Probe synthesis and RNase protection assays were carried out using the Ambion RNase protection assay kit following the manufacturer's instructions

Table 1 Oligonucleotide primers used for mutational analysis of *CDX2*

Name	Oligonucleotide sequence (5' to 3')
CDX2-0039F	ATGGTGAGGTCTGCTCCC
CDX2-0048F	TCTGCTCCCGGACCCTCG
CDX2-0252F	GGCCATCCTGGCCGGCA
CDX2-0413F	CACCACCCGCATCACCAC
CDX2-0604F	AGTGAAAACCAGACGAAG
CDX2-1035R	ATTGCTCTGCCGCTGCAG
CDX2-1047R	GCTCAGCCTGGAATTGCTC

pathway, will therefore require additional cellular factors that directly affect *CDX2* expression. In rare cases, such as in RKO, the APC/ β -catenin pathway is intact and neoplastic growth may result through one or more direct 'hits' within APC-regulated genes, including *CDX2*. Although simple dosage effects, complete loss of function, or unrealized dominant negative effects cannot be ruled out for some *CDX2* mutations, they cannot readily account for the tumor development in the *CDX2*^{+/-} mice (Chawengsaksophak *et al.*, 1997). One possible explanation for the mouse observations and the alteration observed in RKO is that the effects of a *CDX2* alteration may be exacerbated by interruption of a positive feedback loop which normally controls its expression. The resulting reduced expression of

CDX2 could be expected to compromise the normal process of terminal differentiation in the colonic epithelium, given that *CDX2* can induce differentiation of intestinal epithelial cells (Suh and Traber, 1996). This scenario is consistent with the published data in humans and rodents, including the observations that intestinal adenomas of *Apc* mutant mice (Chawengsaksophak *et al.*, 1997), chemically induced rodent tumors (Ee *et al.*, 1995) and human colorectal tumors (Ee *et al.*, 1995, Mallo *et al.*, 1997) have abnormally low levels of *CDX2* expression.

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References

- Aberle H, Bauer A, Stappert J, Kispert A and Kemler R. (1997). *EMBO J.*, **16**, 3797–3804.
- Brattain MG, Levine AE, Chakrabarty S, Yeoman LC, Willson JK and Long B. (1984). *Cancer Metastasis Rev*, **3**, 177–191.
- Chawengsaksophak K, James R, Hammond VE, Kontgen F and Beck F. (1997). *Nature*, **386**, 84–87.
- Ee HC, Erler T, Bhathal PS, Young GP and James RJ. (1995). *Am. J. Pathol.*, **147**, 586–592.
- Eshleman JR, Lang EZ, Bowerfind GK, Parsons R, Vogelstein B, Willson JK, Veigl ML, Sedwick WD and Markowitz SD. (1995). *Oncogene*, **10**, 33–37.
- Fodde R, Edelmann W, Yang K, van Leeuwen C, Carlson C, Renault B, Breukel C, Alt E, Lipkin M, Khan PM and Kucherlapati R. (1994). *Proc. Natl. Acad. Sci. USA*, **91**, 8969–8973.
- He T-C, Sparks AB, Rago C, Hermeking H, Zawel L, da Costa LT, Morin PJ, Vogelstein B and Kinzler KW. (1998). *Science*, **281**, 1509–1512.
- He TC, da Costa LT and Thiagalingam S. (1997). *Bioessays*, **19**, 551–555.
- Kinzler KW and Vogelstein B. (1996). *Cell*, **87**, 159–170.
- Korinek V, Barker N, Morin PJ, van Wichen D, de Weger R, Kinzler KW, Vogelstein B and Clevers H. (1997). *Science*, **275**, 1784–1787.
- Mallo GV, Rechreche H, Frigerio JM, Rocha D, Zweibaum A, Lacasa M, Jordan BR, Dusetti NJ, Dagorn JC and Iovanna JL. (1997). *Int. J. Cancer*, **74**, 35–44.
- Morin PJ, Vogelstein B and Kinzler KW. (1996). *Proc. Natl. Acad. Sci. USA*, **93**, 7950–7954.
- Morin PJ, Sparks AB, Korinek V, Barker N, Clevers H, Vogelstein B and Kinzler KW. (1997). *Science*, **275**, 1787–1790.
- Munemitsu S, Albert I, Souza B, Rubinfeld B and Polakis P. (1995). *Proc. Natl. Acad. Sci. USA*, **92**, 3046–3050.
- Powell SM, Zilz N, Beazer-Barclay Y, Bryan TM, Hamilton SR, Thibodeau SN, Vogelstein B and Kinzler KW. (1992). *Nature*, **359**, 235–237.
- Orford K, Crockett C, Jensen JP, Weissman AM and Byers SW. (1997). *J. Biol. Chem.*, **272**, 24735–24738.
- Riggins GJ, Markowitz S, Wilson JK, Vogelstein B and Kinzler KW. (1995). *Cancer Res.*, **55**, 5184–5186.
- Rubinfeld B, Souza B, Albert I, Muller O, Chamberlain SH, Masiarz FR, Munemitsu S and Polakis P. (1993). *Science*, **262**, 1731–1734.
- Rubinfeld B, Albert I, Porfiri E, Fiol C, Munemitsu S and Polakis P. (1996). *Science*, **272**, 1023–1025.
- Rubinfeld B, Robbins P, El-Gamil M, Albert I, Porfiri E and Polakis P. (1997). *Science*, **275**, 1790–1792.
- Smith KJ, Johnson KA, Bryan TM, Hill DE, Markowitz S, Willson JK, Paraskeva C, Petersen GM, Hamilton SR, Vogelstein B and Kinzler KW. (1993). *Proc. Natl. Acad. Sci. USA*, **90**, 2846–2850.
- Sparks AB, Morin PJ, Vogelstein B and Kinzler KW. (1998). *Cancer Res.*, **98**, 1130–1134.
- Su LK, Kinzler KW, Vogelstein B, Preisinger AC, Moser AR, Luongo C, Gould KA and Dove WF. (1992). *Science*, **256**, 668–670.
- Su LK, Vogelstein B and Kinzler KW. (1993). *Science*, **262**, 1734–1737.
- Suh E, Chen L, Taylor J and Traber PG. (1994). *Mol. Cell. Biol.*, **14**, 7340–7351.
- Suh E and Traber PG. (1996). *Mol. Cell. Biol.*, **16**, 619–625.
- Wicking C, Simms LA, Evans T, Walsh M, Chawengsaksophak K, Beck F, Chenevix-Trench G, Young J, Jass J, Leggett B and Wainwright B. (1998). *Oncogene*, **17**, 657–659.